Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

Dorian S. Houser
National Marine Mammal Foundation
2240 Shelter Island Drive, #200
San Diego, CA 92107
phone: (877) 360-5527 ext.112 fax: (877) 773-3153 email: dorian.houser@nmmpfoundation.org

Samuel Wasser
University of Washington
Seattle, WA 98195
phone: (206) 543-1669 fax: (206) 221-5253 email: wassers@u.washington.edu

John F. Cockrem
Massey University
Palmerston North, New Zealand
phone: +64 (646) 350-4483 fax: +64 (646) 350 5636 email: J.F.Cockrem@massey.ac.nz

Nick Kellar
Southwest Fisheries Science Center
La Jolla, CA 92037
phone: (858) 546-7090 email: Nick.Kellar@noaa.gov

Tracy Romano
Mystic Aquarium and Institute for Exploration
Mystic, CT
phone: (858) 546-7090 email: Nick.Kellar@noaa.gov

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LONG-TERM GOALS

Quantifying physiological indicators of stress in wild marine mammals and the interrelationships between different stress markers can be used to estimate the impact of anthropogenic stressors on marine mammal populations. The United States Navy, as part of its environmental stewardship, can utilize stress markers to assess the acute and chronic impacts that its actions might have on marine mammals. This approach would permit better mitigation of potential impacts and ensure that Navy activities do not come at a deleterious cost to marine mammal populations.

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OBJECTIVES

The objectives of this effort are to: 1) determine the variation in corticosteroid hormones, thyroid hormones, and catecholamines within a dolphin population relative to seasonality, time of day, gender, age and reproductive state; 2) assess relationships between serum corticosteroid levels and levels found in other matrices (i.e. biological samples), including feces and blubber; 3) and to perform adrenocorticotropic hormone (ACTH) and thyrotropin-releasing hormone (TRH) challenges to characterize the activation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axes across multiple matrices, respectively.

APPROACH

Task 1 – Seasonal variations in hormones across multiple matrices
Regular sampling from different matrices (e.g. blubber, blood, feces) will be collected from the U.S. Navy Marine Mammal Program (MMP) dolphin population over the course of a year. Subject dolphins will be split into categories based upon age and will be sampled bi-weekly throughout the year for blood and feces. A subset of animals will be selected for monthly blubber biopsies.

Blood and fecal samples will be collected from dolphins through their voluntary participation. Blood collections will be made from the ventral fluke from the arteriovenous plexus and collections will be made between 0700-1000. Fecal samples will be collected by use of a suction catheter inserted into the anal orifice of the dolphin the day after the blood collection. Bimonthly blubber biopsies will be collected with a 16g tissue biopsy needle.

Serum samples will be processed for adrenocorticosteroids, catecholamines, and thyroid hormones via radioimmunoassay (RIA). The methods have been validated for cortisol and aldosterone in this species (Houser et al., 2011). Parallel processing of serum catecholamines will be performed via high-performance liquid chromatography (HPLC) to assess variability in the measurement methods. Metabolites of cortisol, aldosterone and thyroid hormone will be extracted from fecal samples and measured via RIA using established techniques (Wasser et al., 2010). A multi-step biphasic organic solvent extraction will be used to isolate the corticosteroids from the blubber tissue (Kellar et al., 2009). The hormones will be measured using a commercially available enzyme immunoassay (EIA) and parallel processing via HPLC will be used to verify method performance.

Task 2 – Diurnal variation in hormone production
Hormones will be assessed for diurnal variation during the second year of the study. Ten dolphins will be selected for repeat testing throughout the year. Blood samples will be collected from the dolphins at monthly intervals via voluntary venipuncture of the arteriovenous plexus on the ventral fluke. Samples will be collected first thing in the morning (~0700), at noon, and in the late afternoon (~1700). Blood samples will be processed via RIA and HPLC as described under Task 1. Similar analyses will be conducted on serially collected scat of these 10 individuals over the same 24 hr period and a second 24 hr period one week later when not being sampled for blood.

Task 3 – Adrenocortical sensitivity
Adrenocortical sensitivity and the relationship between activation of the HPA axis and reflection of this activation in serum and other matrices will be determined by submitting five dolphins to an out-of-water stress test. Dolphins will be beached and blood samples and blubber biopsies collected over a two-hour period, and blood samples collected for an additional two hour period once the dolphin is

placed back in the water. Blood and fecal samples will be collected two days prior and two days following the procedure to compare changes vs. baseline and to characterize the recovery period. (Note* The original study design for assessing adrenocortical sensitivity involved the administration of ACTH slow-release gel. However, pilot results indicated that the gel did not reliably induce an adrenocortical response, but that the out-of-water procedure associated with it did produce an adrenocortical response. The study design was modified accordingly.)

A pilot study will also be conducted in which a dolphin is fed fish containing cortisol pellets. Voluntary blood samples will be collected across multiple days and different time frames to determine if serum cortisol levels are elevated and sustained. Based on the results of the pilot, a final dosage for use in five additional subjects will be determined. Procedures will be coupled to blubber biopsies so cortisol deposition in the blubber can be assessed.

Task 4 – Thyroid challenges
Up to three dolphins will be given an exploratory TSH challenge to determine the optimal dosing and sampling schedule. A pre-test blood draw will be collected from the dolphin while it is in its enclosure. The dolphin will then be removed from the water to a location on the pier that is deemed suitable for the procedure by the attending veterinarian. A bolus injection of of TRH will be intramuscularly administered. Blood samples will then be collected every 15 minutes for a period of one hour. Following completion of the sampling period, the dolphin will be returned to its enclosure and voluntary blood samples collected hourly for up to three hours. Following the pilot study, five individuals will be submitted to the TSH challenge at dosages determined in the pilot study. Baseline blood and fecal samples will be collected prior to the first injection and blood collections will be performed as described for the pilot studies. Fecal samples will be collected for daily for four days following the injection. (Note* This protocol was modified from the original in response to a recent publication of a TSH challenge in a single dolphin.)

WORK COMPLETED

Task 1 – Seasonal variation in stress hormones
A group of 30 bottlenose dolphins was identified from within the MMP population that could provide voluntary biweekly blood and fecal samples over a period of a year. The following distribution of animals was obtained:

Age (yrs)	Male	Female
5-15	6	4
16-25	3	3
25+	7	7

Monthly blubber biopsies were collected on the same day of the blood collections in four dolphins.

A total of 735 blood collections were made out of a total of 778 possible draws (~94% success rate). A total of 638 matched fecal samples were collected such that 87% of the blood samples had matched fecal comparisons. Blubber biopsies were collected approximately 12-14 cm below the posterior insertion of the dorsal fins using a 16g biopsy needle. Two to three biopsies were taken each sample

period to ensure that sufficient blubber was obtained for analysis. A total of 47 blubber biopsies were collected over the course of the study.

Sample collection on this task is complete and all blood samples have been analyzed. Approximately 83% of the blubber samples have been processed and all samples for HPLC analysis and fecal hormone analysis are complete.

Task 2 – A total of 357 out of 360 possible blood draws were completed (99%) and a total of 215 out of a possible 240 fecal samples were collected (90%). Blood sample processing is complete for all hormones except aldosterone, epinephrine, and norepinephrine. Fecal sample processing is complete for metabolites of cortisol, aldosterone, and T3.

Task 3 – All cortisol feeding trials are complete. Based on the results of pilot studies, the dose of cortisol was elevated to 60 mg every six hours over a period of five days. Daily voluntary blood and fecal samples were collected and blubber biopsies were taken prior to cortisol feeding (day 0) and on the third and fifth day of feeding. Blood samples have been processed for all of the hormones except aldosterone. Fecal and blubber hormones are currently being processed.

Pilot studies with ACTH administration were completed in the winter and spring of 2013/2014. The protocol was changed in the spring and stress tests were performed throughout the summer with completion in August 2014. Blood samples from the study were processed for ACTH, cortisol, and aldosterone and are currently being processed for the remaining hormones.

RESULTS

Preliminary results indicate that dolphins at the MMP produce low levels of corticosteroids but do not suffer from adrenal exhaustion or insufficiency. In many instances, levels are sufficiently low that alternative means of processing are being employed to accurately assess circulating levels. The low circulating levels likely reflect the lack of predatory and foraging stressors as well as disease mitigation. An incidental finding of megesterol acetate (MegAce) administration impeding cortisol production was found in the MMP population. Due to the widespread use of megesterol acetate, this incidental finding has significant ramifications for the welfare of dolphins under human care.

Significant seasonal differences were noted in free and bound T3. Interactions between seasonality, age, and gender significantly affected all other hormones measured, e.g. ACTH was significantly higher in the spring than other times of the year, but only in males. Cortisol levels were highest in the oldest males (>27 years) and cortisol was signficantly higher in the morning in both sexes than it was in either the evening or at noon.

The biological half-life of cortisol was calculated from the study to be ~109 minutes. Determination of the biological half-life of cortisol permits cortisol levels observed in wild and captive animals to be placed in better context of collection methods (e.g. impact of handling) and provides insight on the kinteics of this corticosteroid. The “stress test” produced marked increases in cortisol and aldosterone to levels in excess of levels commonly observed in wild-caught animals and comparable to or greater than a prior study utilizing ACTH administration to induce cortisol release in dolphins (Thomson and Geraci, 1986; Figure 1). The pilot studies showed that cortisol and aldosterone increase in parallel, and the magnitude of the change in aldosteone may indicate it as a better indicator of stress than cortisol. Preliminary evidence suggests that elevations in cortisol and aldosterone are associated with

suppression of ACTH secretion and free T3 levels. Fecal cortisol closely follows serum cortisol levels over the course of hours, which is more rapid than observed in other mammalian systems. Fecal cortisol may provide a better indicator of the state of stress in a wild-caught dolphin that must experience handling stress prior to the collection of blood samples, i.e. it will reflect the state of the animal several hours prior to capture.

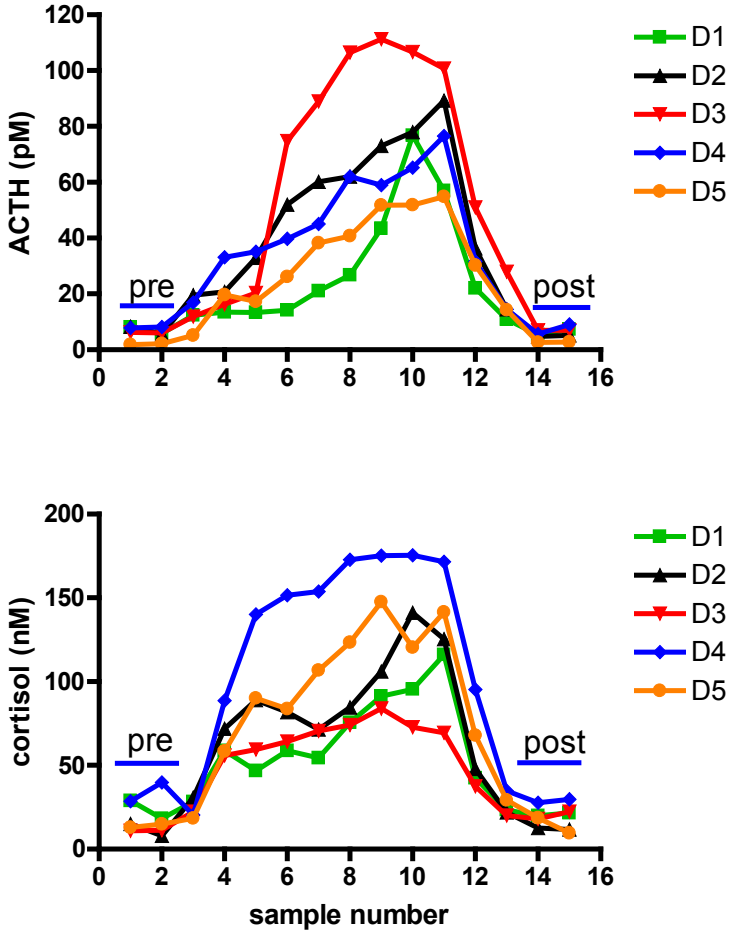

Figure 1. Change in ACTH and cortisol following a stress test in five bottlenose dolphins. The pre- and post- periods are values from blood samples collected the days prior to and following the test (samples 1 and 2, and samples 14 and 15, respectively). ACTH and cortisol rise rapidly after the stress test begins (sample 3), and decline rapidly once the test is concluded and the dolphin is returned to the water (samples 12 and 13, which are one and two hours after the test is concluded). The greatest changes in ACTH did not necessarily correlate with the greatest changes in cortisol.

IMPACT/APPLICATIONS

The ability to identify stress markers relative to monitoring the health of marine mammal populations is critical to understanding the impact of anthropogenic activities upon those populations. The baseline characterization of stress marker variation in dolphins as a function of seasonality, gender, age, and reproductive status is important to assessing measurements made in wild dolphins. Information on levels and dynamics of stress markers between different matrices will provide better estimates of the

overall condition of marine mammals sampled in the wild from either blubber biopsies or fecal collections. In addition, an understanding of the function of the HPA and HPT axis will provide fundamental information on the stress response in these marine mammals, which may differ significantly from that of the terrestrial mammals from which most of our understanding is based. The incidental finding of the impact of MegAce on the dolphin endocrine system has broad-scale implications for the welfare of dolphins under human care.

RELATED PROJECTS

Project: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins (PI Pat Fair)
This project looks at numerous markers of stress in a wild population of marine mammals and compares them to animals under managed care in order to quantify and qualify the impact of environmental stressors on wild dolphins. The dolphins under managed care are from the Georgia Aquarium and the Navy Marine Mammal Program. Ten of the dolphins used in Task 1 of the current study (PI – Houser) were used as the semi-domesticated comparison.

Project: Validating the Novel Method of Measuring Cortisol Levels in Cetacean Skin by Use of an ACTH Challenge in Bottlenose Dolphins (PI Thea Bechsøft)
This project looks to characterize cortisol in the sloughed skin of odontocete cetaceans. Five dolphins used in the stress test of the current study (PI – Houser) were used for the sloughed skin collections.

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PUBLICATIONS

Houser, DS, Champagne, CD, Crocker, DE, Kellar, NM, Cockrem, J, Romano, T, Booth, RK and Wasser, SK. Natural variation in stress hormones, comparisons across matrices, and impacts resulting from induced stress in the bottlenose dolphin. In: *Effects of Noise on Aquatic Life II*, Popper, A and Hawkins, A, eds. [In Press].